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Intracellular Ca^{2+} transients induced by high external K^+ and tetracaine in cultured rat myotubes

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Abstract — Cultured myotubes from rat neonatal skeletal muscle were used to measure intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and membrane potentials (V_m) using the Indo-1 microfluorimetry method and the nystatin perforated membrane patch technique, respectively. Sudden increases in external $[K^+]_o$ from 5 mM to either 22, 42 or 84 mM elicited transient elevations in $[Ca^{2+}]_i$ from a resting level of 106.2 ± 10.3 nM (n = 41) to peak values of 297, 409 and 454 nM, respectively. V_m changes induced by elevated $[K^+]_o$ followed the Nernst equation for $[K^+]_o$. The complex Ca^{2+} release response induced by elevated $[K^+]_o$ can be described by a minimal model involving two components with different kinetics. This analysis revealed that the extent of the Ca^{2+} release by the fast component bears a sigmoidal relationship with V_m (midpoint at −47.5 mV and an effective valence of 4). Furthermore, while the fast transitory component was rather insensitive to $[Ca^{2+}]_o$ and nifedipine, the slow component was profoundly inhibited by the dihydropyridine (10 μM) both in normal and in a Ca^{2+} deficient medium. Tetracaine (0.05 to 2 mM), a blocker of the charge movement associated with excitation-contraction (E–C) coupling, elicited a fast elevation in $[Ca^{2+}]_i$ followed by a rise at a constant rate to levels as high as 1–2 μM, and the changes in $[Ca^{2+}]_i$ were readily reversible. Simultaneous measurements of V_m and $[Ca^{2+}]_i$ suggest that the fast component is coupled to the rapid depolarization of the membrane induced by the anesthetic. We concluded that tetracaine triggers the release of Ca^{2+} from internal stores by at least two different mechanisms, one of which is associated with the depolarizing effects of the drug.

The chain of events leading to the release of Ca^{2+} from the terminal cisternae of the sarcoplasmic reticulum (SR) in vertebrate twitch skeletal muscle begins with the action potential, which is propagated along the transverse tubular membrane (T tubules). The exact link between the membrane potential changes in the T tubules [1] and Ca^{2+} release from the SR remains unresolved [2].

Mammalian skeletal muscle cells in culture are very suitable to study E–C coupling. In this respect, cultured muscle cells from neonatal dysgenic mice, which are devoid of dihydropyridine receptors [3,4], have been used to determine the role of voltage sensors in E–C coupling [2,5].

In fast twitch skeletal muscle elevated $[K^+]_o$ elicits a rise in tension followed by relaxation along an initial plateau phase and a subsequent phase of rapid relaxation [6]. Although membrane depolarization persists in high $[K^+]_o$ [7], the transitory release of Ca^{2+} during the high $[K^+]_o$ contracture probably reflects the physiological E–C coupling mechanism. In the present work we studied $[Ca^{2+}]_i$ transients evoked by a sudden elevation in $[K^+]_o$ in rat myotubes. Our studies show that the $[Ca^{2+}]_i$ transients can be analyzed in terms of two components with different kinetics and sensitivity to nifedipine. We also studied the effect of tetracaine, a local anesthetic known to block the component of the charge movement associated with Ca^{2+} transients and E–C coupling [8,9], and high $[K^+]_o$ contractures [10]. Contrary to what has been described for adult frog muscle fibers, we found that tetracaine induced release of Ca^{2+} by mechanisms which are, at least in part, dependent on membrane potential.

Materials and methods

Cell cultures

Neonatal rat myotubes kept in culture were used to record membrane potential and intracellular free calcium concentration. Primary muscle cultures were prepared and enriched for muscle cells, essentially as described by Daniels [11]. Briefly, minced tissue from the hind limbs of neonatal rats was treated with collagenase for 15 min at 37°C. The suspension was filtered through lens tissue paper and pelleted at low speed. Cells were plated on round (1 cm in diameter) glass coverslips at a density of ca. 350×10^3 per dish (35 mm). Culture medium was F12 plus 10% bovine serum and 2.5% fetal calf serum. To arrest fibroblast growth cytosine arabinoside (50 nM) was added for 24 h two days after plating. Culture media was changed every 3 days afterwards. Myoblast fusion was essentially complete after 4 days.

Calcium measurements and calibrations

Measurements of $[Ca^{2+}]_i$ in cultured myotubes were made using a fluorescence inverted microscope (Diaphot-TMD, Nikon Corp., Japan) provided with two beam splitter-filter cassettes. One beam splitter was used to send the excitation light (355 ± 10 nm) to the cells and the fluorescent light emitted by Indo–1 (acid) inside the cells (> 400 nm) to the second cassette. The second dichroic mirror was used to split the fluorescent light into beams of light centered at 410 and 485 nm (± 5 nm), respectively. The intensity of the light at each wavelength was continuously measured using a photomultiplier. The output from each photomultiplier was sampled (at either 0.36 or 1.0 s/sample) using a unit equipped with a buffer amplifier, a 12-bit analog-to-digital converter, a microprocessor, and a serial port output (Photometer P1, Nikon). Digital data from both photometers were captured simultaneously and processed using a microcomputer (Compaq, 386/25) by means of two serial ports. The data acquired with this system represent the normalized fluorescence (F) emitted at 410 and 485 nm (0–100%). A computer program calculated the F_{410nm}/F_{485nm} ratio, obtained the corresponding $[Ca^{2+}]_i$ from a calibration curve, and displayed the time course of F_{410nm}, F_{485nm} and $[Ca^{2+}]_i$. To calibrate the system we prepared different Ca^{2+}-buffered solutions (in mM: 5 Na-EGTA, 140 KCl, 10 Na-HEPES, pH 7.0) by adding different amounts of $CaCl_2$. The final $[Ca^{2+}]_o$ was calculated and measured using a Ca^{2+} electrode (CAL-1, WPI Instruments, Inc., New Haven, CO, USA). Measurements of F_{410nm} and F_{485nm} values needed to calculate the F_{410nm}/F_{485nm} ratio at each $[Ca^{2+}]_o$ were carried out inside the same chamber used for the experiments. For each Ca^{2+}-buffered solution (1 ml), we added 0.025 ml of a 25% ethanol solution containing the acid form of Indo–1 and proceeded to measure the corresponding F_{410nm} and F_{485nm} values. Cells were loaded with Indo–1/AM (2 μM) at room temperature (20–25°C) for 1–2 h in a modified Krebs solution of the following composition (in mM): 145 NaCl, 5 KCl, 2.6 $CaCl_2$, 1 $MgCl_2$, 10 Na-HEPES, 5 $NaHCO_3$, 5.6 glucose, pH = 7.4 containing pluronic acid (0.02%). Loading the cells in the absence of pluronic acid for 1–2 h, although less effective in terms of the intracellular concentration of the dye achieved after the incubation period, produced cells which gave similar responses to elevated $[K^+]_o$. The composition of the Ca^{2+}-deficient solution was that of the modified

Krebs saline (no $CaCl_2$ added) plus NaEGTA (6 mM at pH 7.4).

For intracellular calcium measurements, the cover slips were mounted in a perifusion chamber. The cells were continuously superfused (0.2 ml/s) with a modified Krebs solution, and drugs were applied at the doses indicated in the figures using the same perifusion system. The exchange time was estimated at about 2 s by measuring the response to a rapid elevation in $[K^+]_o$.

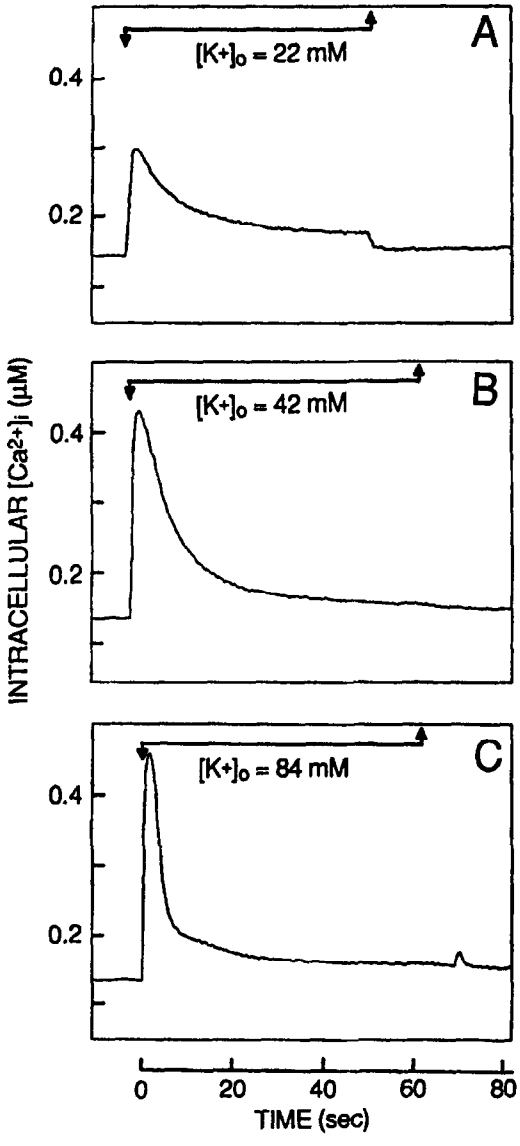

Fig. 1 High $[K^+]_o$-induced $[Ca^{2+}]_i$ transients. $[Ca^{2+}]_i$ rise in response to either (A) 22, or (B) 42 or (C) 85 mM $[K^+]_o$ from mature myotubes. The records are representative of 3–4 experiments for each condition. V_m values measured in parallel experiments were −41.6, −28.1 and −13 mV for (A), (B) and (C), respectively.

In the studies presented here we selected those cells in which high $[K^+]_o$ (42 mM) elicited a measurable $[Ca^{2+}]_i$ rise. Similar $[Ca^{2+}]_i$ responses were obtained from either single isolated myotubes or from single myotubes forming part of small cell aggregates.

Simultaneous recordings of membrane potential and $[Ca^{2+}]_i$

Membrane potential (V_m) was recorded under current clamp conditions using nystatin (100 μg/cm^3) perforated membrane patch procedure. Membrane potential V_m measurements were carried out using an EPC-7 patch clamp amplifier. To record simultaneously V_m and $[Ca^{2+}]_i$ from the same myotube, the output from the photomultipliers detecting the 405 and 485 nm signals was fed to an analog voltage divider. The output from this circuit, which is proportional to $[Ca^{2+}]_i$ and the V_m output from the patch clamp amplifier were recorded on magnetic tape using a 2 channel VCR/PCM system (Unitrade Digital-4, Toshiba).

Chemicals and reagents

Indo-1/AM, Indo–1 acid and pluronic acid were obtained from Molecular Probes (Eugene, OR, USA). Nifedipine was from Research Biochemicals Incorporated (RBI, Natick, MA, USA) and tetracaine was from Sigma (Chemical Co., St Louis, MO, USA).

Results

A sudden elevation in $[K^+]_o$ elicits a biphasic $[Ca^{2+}]_i$ rise

In the presence of physiological Krebs saline ($[K^+]_o$ = 5 mM) resting $[Ca^{2+}]_i$ in non-stimulated rat myotubes (4–6 days in culture) was 106.2 ± 10.3 nM (mean ± SD; n = 41), and remained unchanged for up to 3–4 h at room temperature. A step increase in $[K^+]_o$ to either 22, 42 or 84 mM always

elicited a transitory $[Ca^{2+}]_i$ rise in mature cultured myotubes. The peak value of $[Ca^{2+}]_i$ was reached in less than 2 s, and thereafter $[Ca^{2+}]_i$ returned to the resting level along a characteristic curve consisting of two phases.

In general, immature myotubes (2–3 days in culture) did not respond to high $[K^+]_o$; only occasionally a small Ca^{2+} signal was observed. A small proportion of spontaneously formed myoballs (7–12 days in culture) responded to high $[K^+]_o$ with a minute Ca^{2+} signal and responded to other Ca^{2+} release drugs including caffeine (10 mM) in a more substantial manner.

To evaluate in a quantitative manner the effects of different experimental parameters, such as Ca^{2+}

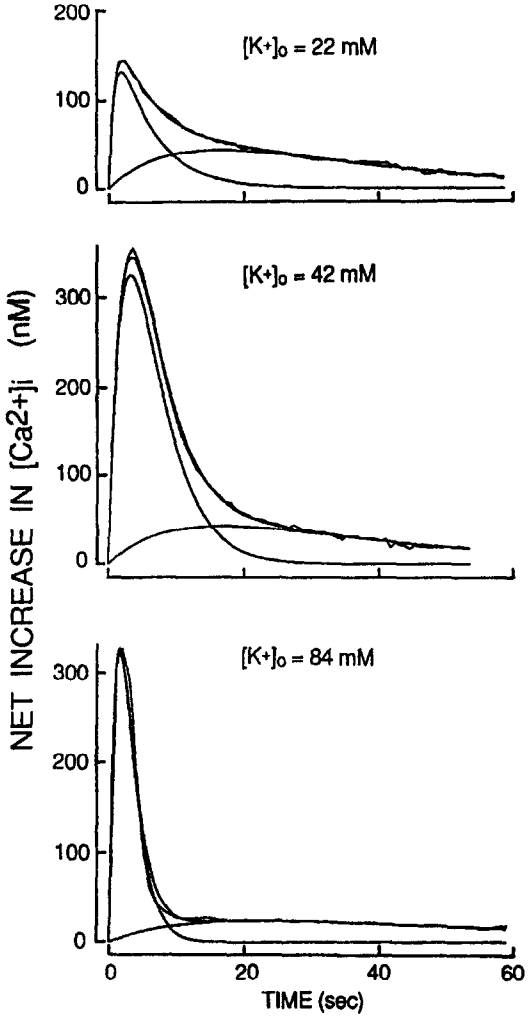

Fig. 2 Kinetic analysis of the time course of $[K^+]_o$-induced $[Ca^{2+}]_i$ transients. Each panel shows 4 superimposed traces representing: the experimental record (noisy), the sum of the model components (best fit), the individual fast and delayed transitory components. Records shown are representative of 3 experiments. Mean values of the model parameters obtained from the least squares regression fit are given in Table, Part A. V_m values as in Figure 1.

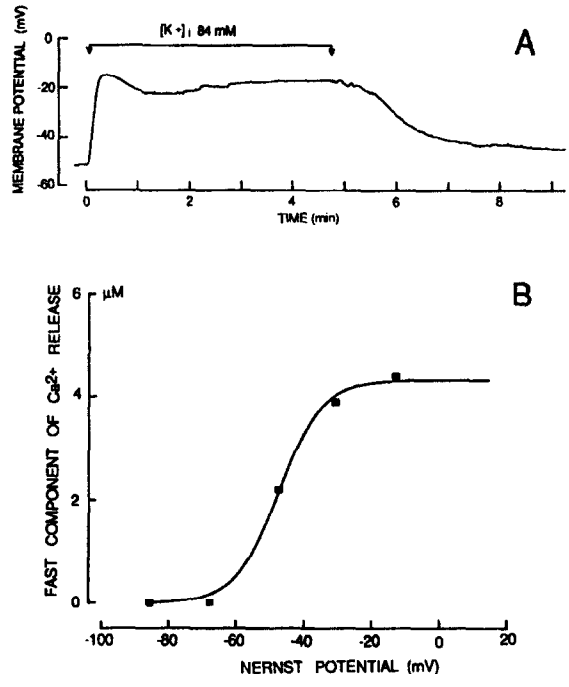

Fig. 3 Membrane potential changes evoked by a rapid elevation of $[K^+]_o$ to: (A) 84 mM; and (B) steady-state distribution of the model estimated maximum $[Ca^{2+}]_i$ due to the fast component. (A) Membrane potential was measured using the nystatin perforated membrane patch technique either simultaneously with $[Ca^{2+}]_i$ (as in Fig. 8) or in parallel experiments (as in Fig. 1). Cultured myotubes were continuously superfused with Krebs solution at a rate of about 0.2 cm^3/s. Solution changes occurred at the times indicated by the arrows. (B) Symbols represent the mean of three $[Ca^{2+}]_f^{max}$ values per $[K^+]_o$ (for details *see* Table, Part A). Point at –85.7 mV (or 5 mM $[K^+]_o$) corresponds to the resting myotube. The Nernst potential in mV at each $[K^+]_o$ was calculated at 22.5°C as:

$$V_m = 58.4 \log_{10}\{[K^+]_o/[K^+]_i\}$$

Taking $[K^+]_i$ as 145 mM we obtained –85.7, –67.8, –47.8, –31.4 and –13.8 mV for 5, 10, 22, 42 and 84 mM $[K^+]_o$, respectively. Some of the actual values measured are given in Figure 1.

removal on these Ca^{2+} release transients, we consider now the following minimal kinetic model that could reproduce the observed time course of the $[Ca^{2+}]_i$ signal.

Kinetic analysis of the $[Ca^{2+}]_i$ transient elicited by high $[K^+]_o$

Figure 2 shows the analysis of the time course of 3 $[Ca^{2+}]_i$ signals evoked by (A) 22, (B) 42 and (C) 84 mM $[K^+]_o$ together with the analysis based on the following model. Assuming that the Ca^{2+} release is made up of two transitory components, it is possible to fit the experimental record (noisy trace) using the empirical function:

$$[Ca^{2+}]_i(t) = [Ca^{2+}]_f^{max} \{1 - \exp(-k_{fr} \cdot t)\} \exp(-k_{fu} \cdot t) + [Ca^{2+}]_d^{max} \{1 - \exp(-k_{dr} \cdot t)\} \exp(-k_{du} \cdot t) \quad \text{(Eq. 1)}$$

where $[Ca^{2+}]_f^{max}$ ($[Ca^{2+}]_d^{max}$) represent the maximal Ca^{2+} concentration that would be achieved by the fast (delayed) process in the absence of Ca^{2+} uptake; k_{fr} (k_{dr}) represents the activation rate constant of the fast (delayed) Ca^{2+} release process; k_{fu} (k_{du}) represents the Ca^{2+} uptake rate constant affecting the fast (delayed) process. The analysis of the Ca^{2+} transients elicited by high $[K^+]_o$ either 22 mM (Fig. 2A), 42 mM (Fig. 2B), or 84 mM (Fig. 2C) reveals that the fast component determines the peak value of the Ca^{2+} release and the delayed component, with a very slow decay, determines the plateau phase.

To verify that the resting membrane potential in our myotube preparation depended on $[K^+]_o$, we measured the changes in V_m induced by rapid elevations in $[K^+]_o$ (Fig. 3A) under zero current clamp conditions. Increasing $[K^+]_o$ from 5 to 84 mM depolarized the myotube from –68 to –15 mV. Increasing $[K^+]_o$ to 42 mM produced an average depolarization to about –28 mV (n = 3). The mean of two other measurements at 22 mM $[K^+]_o$ was about –42 mV. These data indicate that in our preparation of myotubes, V_m roughly follows the Nernst relation for $[K^+]_o$.

Figure 3B shows a graph of the mean values (n = 3) of $[Ca^{2+}]_f^{max}$ as a function of the Nernst potential for $[K^+]_o$ taking the intracellular $[K^+]_i$ as 140 mM. The sigmoidal curve represents the best fit of

Table Kinetic analysis of the $[Ca^{2+}]_i$ signal.

A Parameters in the presence of physiologic 2.6 mM Ca^{2+}

$[K^+]_o$	$[Ca^{2+}]_{i,b}$	$[Ca^{2+}]_f^{max}$	$[Ca^{2+}]_d^{max}$	k_{fr}^{-1}	k_{fu}^{-1}	k_{dr}^{-1}	k_{du}^{-1}	n
[mM]	[μM]			[s]				
10	0.12	0.2	0.3	10.0	6.6	45.4	12.2	2
22	0.14	2.2	1.9	10.0	5.4	25.8	22.2	2
42	0.09	3.9	1.3	15.2	3.2	12.2	28.0	3
84	0.13	4.4	1.1	10.5	2.2	8.9	41.8	3

B Parameters in control and Ca^{2+} deficient medium with or without nifedipine (10 μM); transients elicited by 42 mM $[K^+]_o$

$[Ca^{2+}]_o$	$[nif]_o$	$[Ca^{2+}]_{i,b}$	$[Ca^{2+}]_f^{max}$	$[Ca^{2+}]_d^{max}$	k_{fr}^{-1}	k_{fu}^{-1}	k_{dr}^{-1}	k_{du}^{-1}	n
[mM]	[μM]				[s]				
2.6	10	0.09	6.4	0.1	16.8	1.9	10.3	14.9	2
0	10	0.12	2.7	0.02	14.9	1.8	1.8	28.4	2
0	0	0.10	5.6	0.5	13.7	2.4	12.3	21.6	3

$[Ca^{2+}]_f^{max}$ and $[Ca^{2+}]_d^{max}$ represent the extent of the net increase in $[Ca^{2+}]_i$ by the fast and delayed component, respectively.

the Boltzmann function:

$$[Ca^{2+}]_f^{max}(V_m) = [Ca^{2+}]_f^{max}(V_m = 20mV) \frac{1}{1 + \exp\left(-a\frac{(V-V_o)}{kT}\right)}$$

Eq. 2

to the mean $[Ca^{2+}]_f^{max}(V_m)$ values at 10, 22, 44 and 84 mM $[K^+]_o$ (5 mM was taken as 0). The parameter 'a' represents the 'effective valence' of the voltage sensor responsible for the coupling between the membrane potential V_m and the fast Ca^{2+} release process; V_o is the midpoint potential and $[Ca^{2+}]_f^{max}(V_m = 20\ mV)$ is the maximal Ca^{2+} concentration that would be achieved by the fast process at 20 mV in the absence of Ca^{2+} uptake. The following values were obtained from the best fit: $[Ca^{2+}]_f^{max}(V_m = 20\ mV) = 4.3\ \mu M$, a = 4 elementary charges, $V_o = -47.5$ mV. By contrast the corresponding $[Ca^{2+}]_d^{max}$ values decreased from 1.9 (at 22 mM) to 1.1 μM (at 84 mM $[K^+]_o$) as V_m was made more positive (*see* Table, Part A).

Differential sensitivity to nifedipine and external calcium

The initial $[Ca^{2+}]_i$ rise (Fig. 4A) was moderately sensitive to external $[Ca^{2+}]_o$ (Fig. 4B) and to nifedipine (Fig. 4C). The mean peak value of $[Ca^{2+}]_i$ was 409 nM (n = 3) at physiologic $[Ca^{2+}]_o$ and 350 nM in the absence of Ca^{2+} (n = 3). By contrast, the falling phase of the $[Ca^{2+}]_i$ transient was faster both

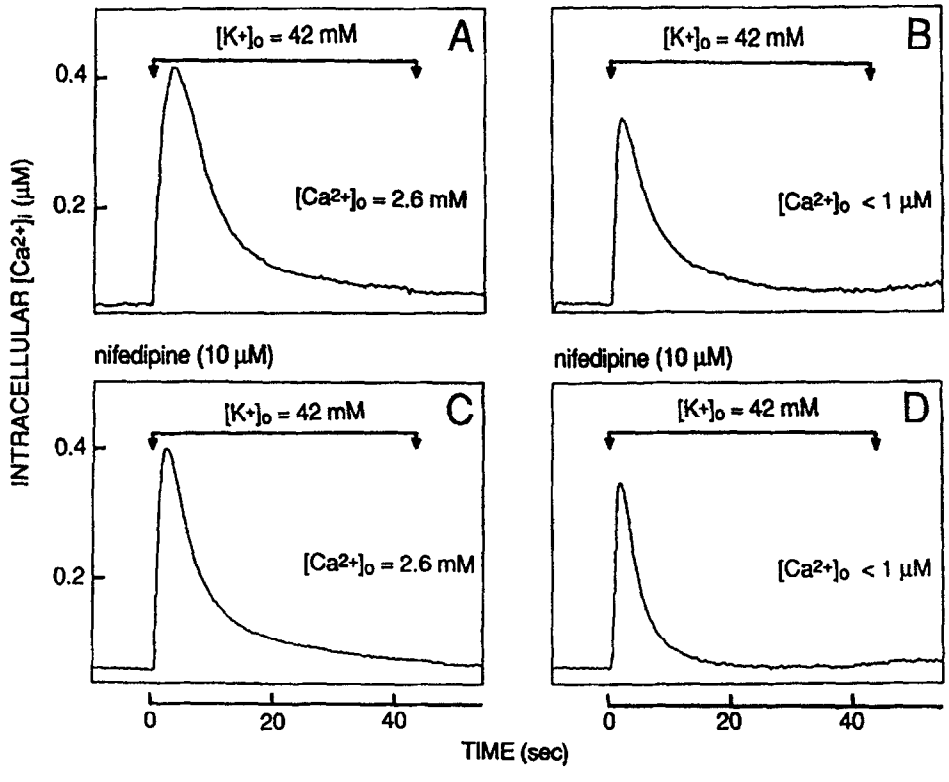

Fig. 4 Effects of low external Ca^{2+}, nifedipine and nifedipine in low external Ca^{2+} on high $[K^+]_o$-induced $[Ca^{2+}]_i$ rise. (B, D) 2–3 minutes prior to the application of the high $[K^+]_o$ solution cells were superfused with Krebs solution containing low $[Ca^{2+}]_o$ (<1μM; NaEGTA added); $[Ca^{2+}]_o$ in the high $[K^+]_o$ solution was <1 μM (NaEGTA added). (C, D) 3–5 minutes prior to the application of either high $[K^+]_o$ (2.6 mM $[Ca^{2+}]_o$) plus nifedipine (10 μM; C) or high $[K^+]_o$ ($[Ca^{2+}]_o$ <1 μM) plus nifedipine (10 μM) cells were superfused with the control medium plus nifedipine. The records are representative of 3 experiments for each condition.

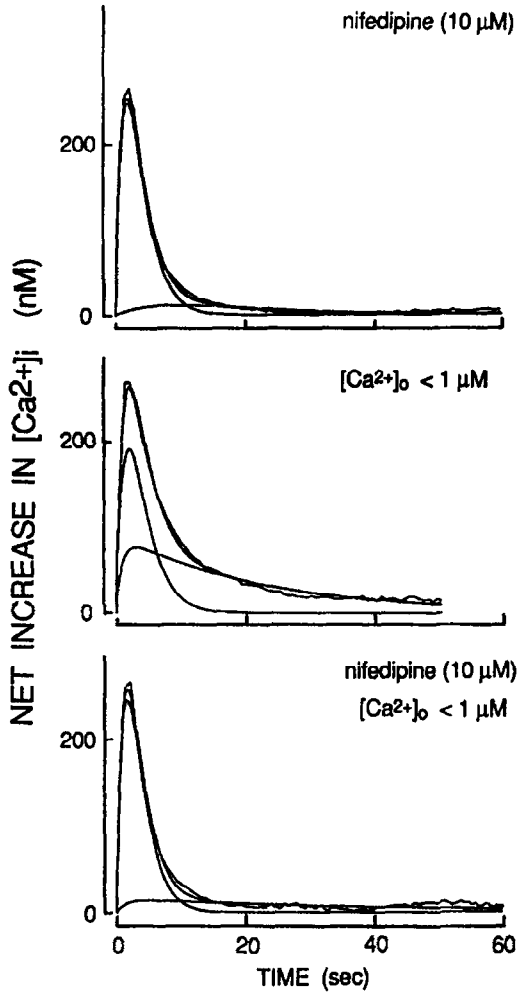

Fig. 5 Model analysis of the effects of low $[Ca^{2+}]_o$ and nifedipine on the components of the $[Ca^{2+}]_i$ signal. Experimental records (noisy trace) following the protocol described in the text and illustrated in Figure 4. Mean values of the model parameters for the best fit of the experimental $[Ca^{2+}]_i$ are given in Table, Part B.

in the Ca^{2+}-deficient solution (6 mM NaEGTA added; Fig. 4B) and in the Ca^{2+}-deficient medium plus nifedipine (10 μM; Fig 4D). Under these conditions the mean peak value of $[Ca^{2+}]_i$ was 300 nM (n = 3). Clearly, either a decreased rate of Ca^{2+} release (entry into the cytosol) or an increased rate of Ca^{2+} uptake (outflow from the cytosol) could explain the observed decrease in amplitude of the $[Ca^{2+}]_i$ transient. For this reason we used again our kinetic model to evaluate the changes.

As illustrated in Figure 5 and Table (Part B), nifedipine but not low $[Ca^{2+}]_o$ inhibits the extent of the net increase by the delayed component ($[Ca^{2+}]_d^{max}$ in Table, Part B) from 0.5 to 0.02 μM in the presence of nifedipine, both experiments in low $[Ca^{2+}]_o$. Furthermore, the time constants of Ca^{2+} uptake (k_{fu}^{-1} in Table, Part B) at 42 mM $[K^+]_o$ were rather insensitive to both nifedipine (1.9 s) and Ca^{2+} deficient conditions (1.8 s). These data led us to conclude that the two components may indeed represent two distinct processes of the E–C coupling in cultured myotubes.

Differential effects of tetracaine and procaine on $[Ca^{2+}]_i$

In rat myotubes tetracaine (0.05–2 mM) but not procaine (2–5 mM) evoked a dose-dependent complex Ca^{2+} release signal (Fig. 6). Tetracaine-induced $[Ca^{2+}]_i$ rises were markedly different from those evoked by high $[K^+]_o$. As illustrated in Figure 6, unlike high $[K^+]_o$-induced $[Ca^{2+}]_i$ transients (Fig. 1), a low dose of tetracaine (0.05–0.2 mM) elicited a marked increase in $[Ca^{2+}]_i$ and Ca^{2+} remained at an elevated level during the entire period of application of the anesthetic (Fig. 6A–C). At higher doses of tetracaine (0.4–2 mM), $[Ca^{2+}]_i$ continued to rise at a higher rate, and could reach levels as high as 1–2 μM (Fig. 6F). Failing to remove the anesthetic caused irreversible damage of the myotubes. Regardless of the concentration of tetracaine used (0.05–2 mM), removal of the drug caused a rapid decrease in $[Ca^{2+}]_i$ towards a minimum level which was above the resting $[Ca^{2+}]_i$. Immediately after this $[Ca^{2+}]_i$ fall, a second transitory $[Ca^{2+}]_i$ rise occurred. The appearance of the 'off' response always occurred at doses of tetracaine equal or greater than 0.15 mM.

The $[Ca^{2+}]_i$ signal elicited by tetracaine (1.6 mM) remained unchanged both in a Ca^{2+} deficient medium or in the presence of nifedipine (10 μM). However, the characteristic off-response which followed the removal of the drug was greatly diminished or even absent in a Ca^{2+} deficient medium. Small $[Ca^{2+}]_i$ oscillations during the off-response were often observed in a low $[Ca^{2+}]_o$ medium (Fig. 7A). The initial component of the $[Ca^{2+}]_i$ signal

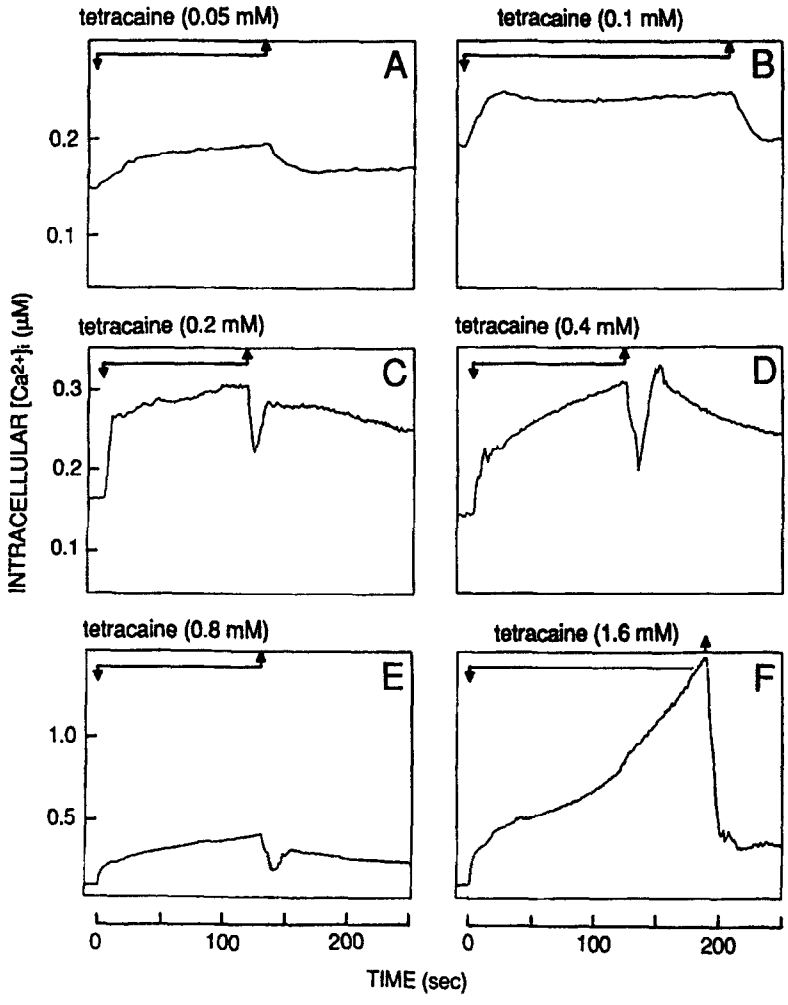

Fig. 6 $[Ca^{2+}]_i$ signals elicited by different doses of tetracaine. Each record (A–F) represents the $[Ca^{2+}]_i$ rise induced by tetracaine at the concentration indicated. Dishes were exposed to the drug only once. Mean V_m prior to the application of the drug was -70.5 ± 1.5 mV ($n = 6$). A low dose (0.3 mM) of tetracaine depolarized the membrane to -24 ± 8 mV ($n = 3$). $[Ca^{2+}]_i$ records are representative of 2–5 experiments for each tetracaine concentration.

elicited by tetracaine (0.8 mM) in the presence of physiologic $[Ca^{2+}]_o$ (Fig. 7B, noisy trace) reveals that the delayed component of the $[Ca^{2+}]_i$ rise is sustained. The fitted curve (dashed line) required a very low k_{dr} value (Eq. 1), as if the Ca^{2+} removal rate k_{dr} was negligible. Assuming that the decay in $[Ca^{2+}]_i$ during the delayed phase is due to Ca^{2+} removal (k_{dr} in Eq. 1) by the SR, we can propose that tetracaine (≥0.4 mM) inhibits Ca^{2+} uptake, probably acting on the Ca^{2+}-ATPase present in the SR.

Simultaneous recordings of V_m (Fig. 8A) and $[Ca^{2+}]_i$ (Fig. 8B) show that tetracaine (0.8 mM) induces a rapid depolarization of the membrane in phase with the onset of the $[Ca^{2+}]_i$ rise. Upon removal of the anesthetic the $[Ca^{2+}]_i$ signal followed the usual biphasic pattern but V_m returned very slowly to the control value of –68 mV. Another ef-

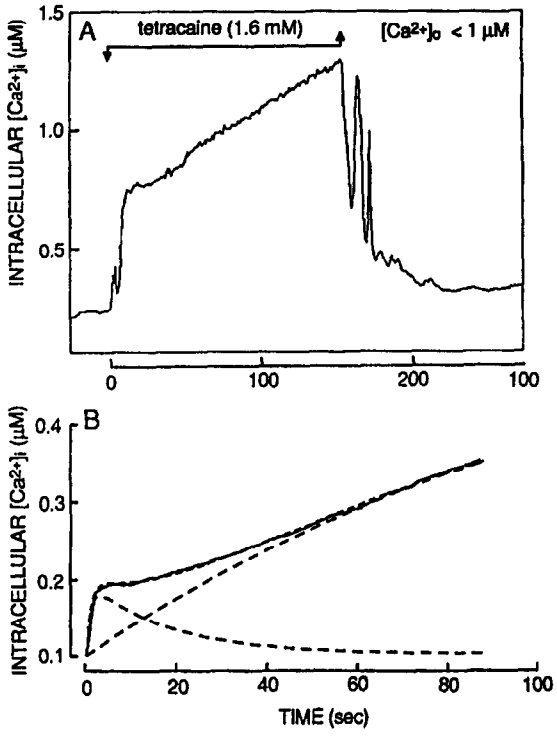

Fig. 7 Tetracaine-induced $[Ca^{2+}]_i$ rise in a $[Ca^{2+}]_o$-deficient medium (A) and different effects on model components of the signal (B). (A) Cells were continuously superfused with a Ca^{2+} deficient medium (no added Ca^{2+} plus 0.1 mM NaEGTA). Tetracaine was applied as indicated. (B) The early part of the $[Ca^{2+}]_i$ signal (noisy trace) induced by tetracaine (0.8 mM; $[Ca^{2+}]_o$ = 2.6 mM) was analyzed using the two component model (dashed curves). The parameters for the best fit were: $[Ca^{2+}]_{i,b}$ = 79 nM, $[Ca^{2+}]_f^{max}$ = 0.1 μM, $[Ca^{2+}]_d^{max}$ = 0.6 μM, k_{fr}^{-1} = 1 s, k_{fu}^{-1} = 18.2 s, k_{dr}^{-1} = 110 s, k_{du}^{-1} = 999 s.

fect of tetracaine is depicted in Figure 8C. After the exposure to tetracaine the myotube fired trains of action potentials.

To further elucidate the role of V_m we studied the effects of tetracaine on $[Ca^{2+}]_i$ in the presence of elevated $[K^+]_o$ (42 mM). As shown in Figure 9 the initial part of the $[Ca^{2+}]_i$ signal in response to a brief exposure to tetracaine (2 mM) was not significantly different to that observed in normally polarized myotubes. In contrast, the off-response which is characterized by the presence of multiple fast $[Ca^{2+}]_i$ transients was absent. This result suggests that the status of V_m during the repolarization of the membrane, following the removal of the drug, determines the off-$[Ca^{2+}]_i$ signal. Holding the membrane potential clamped at either –70 or –80 mV substantially diminished the $[Ca^{2+}]_i$ rise evoked by tetracaine (0.3 mM), and during the application of tetracaine, outward K^+-currents were found to be blocked by the drug in a dose-dependent manner (not shown).

In contrast, myotube resting $[Ca^{2+}]_i$ remained unchanged when procaine (2–5 mM), the other local anesthetic used here, was applied. However, kinetic analysis of the high (42 mM) $[K^+]_o$-induced $[Ca^{2+}]_i$ release recorded in the presence of procaine (2–5 mM) revealed that the extent of the $[Ca^{2+}]_i$ rise dur-

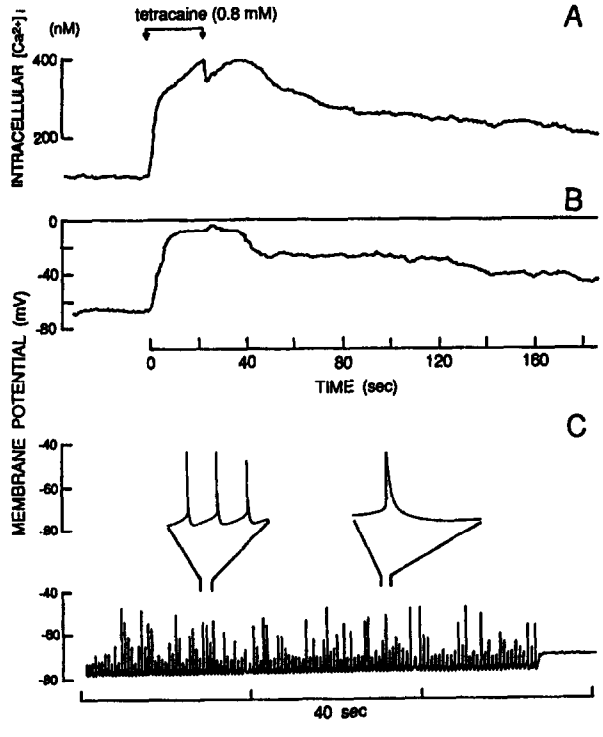

Fig. 8 Simultaneous measurements of $[Ca^{2+}]_i$ and membrane potential V_m. $[Ca^{2+}]_i$ rise (A) and V_m changes (B) in response to tetracaine (0.8 mM). V_m was measured under current clamp conditions (zero holding current). Upon removal of the drug V_m returned to the resting level (3–5 min). After full recovery of V_m had been achieved, a prolonged train of action potentials was spontaneously fired (C, lower record). Individual spikes are depicted (C, upper records) on an expanded time base to show details.

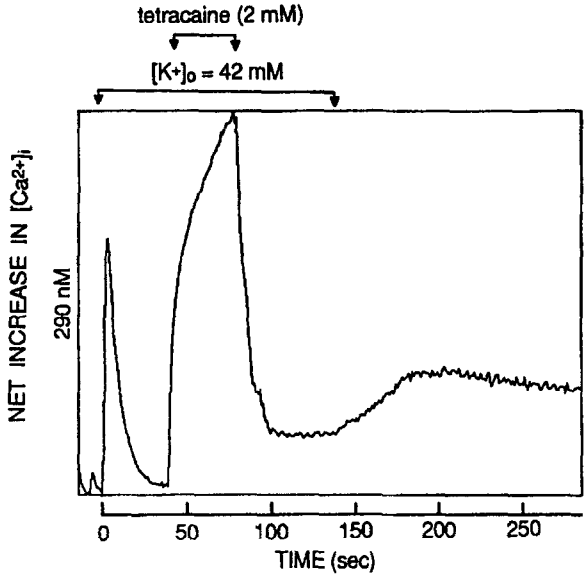

Fig. 9 Effects of tetracaine on a partly depolarized myotube. High $[K^+]_o$ (42 mM) was applied first and cells were exposed to tetracaine (added to the high $[K^+]_o$ solution at 2 mM) at the times indicated by the arrows.

ing the delayed component of the signal ($[Ca^{2+}]_d^{max}$ in the Table) was reduced from 1.3 to 0.12 μM (n = 2). The other parameters of the model including the rate constants k_{fr}, k_{fu}, k_{dr} and k_{du} were not affected in a significant manner.

Discussion

We have shown here that a step increase in external K^+ concentration (≥22 mM) elicited a transitory elevation in $[Ca^{2+}]_i$ in cultured mature myotubes. Model and experimental analysis of the $[Ca^{2+}]_i$ signals show that high $[K^+]_o$ elicits the $[Ca^{2+}]_i$ rise by two kinetically and pharmacologically distinct processes. Only the early component depends on V_m and is insensitive to nifedipine (<20 μM) in a Ca^{2+} deficient medium. In contrast, the delayed component is inhibited by nifedipine and depends on external $[Ca^{2+}]$, and probably is the dihydropyridine sensitive charge movement-Ca^{2+} release mechanism present in rabbit skeletal muscle [12]. The kinetic model also predicts that the extent of the Ca^{2+} release in the absence of uptake might elevate $[Ca^{2+}]_i$ into the micromolar range. We hypothesized that while the parameters of the fast component may be relevant to the $[Ca^{2+}]_i$ release-uptake process during a twitch, the parameters of the delayed component may be relevant to the $[Ca^{2+}]_i$ release-uptake process during tonic contraction. We also found that the local anesthetic tetracaine (0.05–2 mM) but not procaine (2–5 mM) induces a multiphasic release of Ca^{2+} from an internal store which is insensitive to V_m, nifedipine (20 μM) and external $[Ca^{2+}]_o$. Since tetracaine also depolarizes the membrane of the myotubes, we propose that the first phase of the tetracaine-triggered Ca^{2+} signal is due to the depolarization induced by the drug. The second phase of the signal, during which $[Ca^{2+}]_i$ increases at a constant rate, also occurs in the presence of high $[K^+]_o$ (42 mM) suggesting that the process is independent of V_m. Analysis of the second phase using the kinetic model revealed that the drug most likely blocked the uptake of Ca^{2+} (k_{du}). It is also possible that Ca^{2+}-induced release of Ca^{2+} [13,14] is responsible, at least in part, for the off-response observed upon removal of the drug.

Elevated $[K^+]_o$ triggers Ca^{2+} release by two kinetically and pharmacologically distinct processes

In vertebrate skeletal muscle fibers the following signal-transduction steps occur during a twitch caused by a single action potential: (i) inward propagation of the surface membrane action potential along the transverse tubular membrane; (ii) signal transmission across the gap between the T-tubular membrane and the SR terminal cisternae; (iii) Ca^{2+} release from the SR; and (iv) Ca^{2+} uptake by the SR. Probably, these 4 steps are also involved in E–C coupling in high $[K^+]_o$-evoked contractures although there are some marked differences between the $[Ca^{2+}]_i$ signals during a twitch and during a high $[K^+]_o$ contracture. However, we have shown that V_m measured using the nystatin perforated patch, roughly followed $[K^+]_o$ as predicted by the Nernst equation for K^+ in our preparation of myotubes. Thus, a rapid elevation in $[K^+]_o$ depolarizes the myotube membrane (i), and this in turn causes the transitory $[Ca^{2+}]_i$ rise (ii–iv). Since the fast component of this $[Ca^{2+}]_i$ signal was also found to be in-

sensitive to external $[Ca^{2+}]_o$, we may conclude that Ca^{2+} entry is not involved in this component and, therefore, the rate of Ca^{2+} release (iii) from the SR must be greater than the rate of Ca^{2+} uptake (iv).

The extent of the Ca^{2+} release during the fast component (iii), after correction to allow for the uptake (iv), depends on V_m with a midpoint potential at –47.5 mV and an effective valence of 4 elementary charges. The valence of 4 found here is close to the value of 3 for the moving charge in the electro-mechanical model of E–C coupling [15]. We concluded that the mechanisms (i–iv) by which membrane depolarization elevates $[Ca^{2+}]_i$ in cultured myotubes are common to both, the single action potential twitch and the high $[K^+]_o$ contracture.

Resting $[Ca^{2+}]_i$ and high $[K^+]_o$-induced $[Ca^{2+}]_i$ transients similar to those presented here in cultured rat myotubes have been observed before using different Ca^{2+}-sensitive dyes and calibration procedures [16,17]. However, they showed high $[K^+]_o$-induced $[Ca^{2+}]_i$ transients to be dependent on external $[Ca^{2+}]_o$ in contrast to the results presented here. Furthermore, in colchicine-induced myoballs (14–16 days old), these authors also found biphasic $[Ca^{2+}]_i$ transients which were insensitive to external $[Ca^{2+}]_o$ and sensitive to nifedipine. Since in all our experiments we used mature (4–6 days old) myotubes, we may conclude that $[Ca^{2+}]_o$ independent E–C coupling appears around the fourth day in myotubes culture, that is, two days after fusion and expression of several markers [5,18]. While high $[K^+]_o$ triggers biphasic $[Ca^{2+}]_i$ transients in colchicine-induced myoballs we always observed a single fast transitory $[Ca^{2+}]_i$ rise. We have also observed that young myotubes (≤3 days old) do not respond to high $[K^+]_o$ and $[Ca^{2+}]_i$ remains at its resting, non-stimulated level in the presence of elevated $[K^+]_o$. The absence of response or a diminished response to high $[K^+]_o$ in spontaneously formed myoballs (≥7 days old) suggested that the $[Ca^{2+}]_i$ rise in response to membrane depolarization may be regulated during cell differentiation [17], and thus, E–C coupling may be down regulated in older cells.

In addition to the differences in kinetics, the two components in our model exhibited differential sensitivity to nifedipine, i.e. the drug markedly inhibited only the delayed component. Since dihydropyridines block both T-tubules Ca^{2+} currents and charge movement in vertebrate muscle fibers [19], and interfere with the development of tension in cultured skeletal muscle [20], it is difficult to draw conclusions as to the physiological role of the delayed component of the $[Ca^{2+}]_i$ signal present in mature myotubes.

Differential effects of local anesthetics on $[Ca^{2+}]_i$

We have shown here that tetracaine (0.1–2 mM) but not procaine (2–5 mM) can depolarize the membrane and evoke a biphasic $[Ca^{2+}]_i$ rise in mature rat myotubes. Upon removal of the drug the membrane slowly repolarized. The $[Ca^{2+}]_i$ signal during repolarization of the myotube was characterized by an initial rapid decrease in $[Ca^{2+}]_i$ followed by a marked second $[Ca^{2+}]_i$ rise (Fig. 6). Application of tetracaine (2 mM) to myotubes partly depolarized by elevated $[K^+]_o$ (Fig. 9) elicited $[Ca^{2+}]_i$ increases which, although similar to those observed when the myotube was allowed to depolarize, were not identical. Among the differences between the two responses we found that the onset of the $[Ca^{2+}]_i$ rise induced by tetracaine was slower in depolarized myotubes. In addition, under these conditions, removal of the drug induced only the rapid decrease in $[Ca^{2+}]_i$ and the second $[Ca^{2+}]_i$ rise (off-response) was absent from the records. The $[Ca^{2+}]_i$ signal probably represents the predominant component of the tetracaine-induced $[Ca^{2+}]_i$ signal which may be due to a direct action on the mechanisms of Ca^{2+} release or/and Ca^{2+} uptake.

In vertebrate muscle tetracaine blocks the delayed component of the charge movement [8,21], thought by some to result from the Ca^{2+} release process itself [9], at several different sites. This earlier work already suggested that there are tetracaine sites in the system of surface membranes of the muscle fiber. We also noticed that tetracaine reversibly blocked all components of the outward K^+-currents. As to the mechanism by which tetracaine depolarizes the myotube, the simplest explanation for the depolarization evoked by tetracaine is that the local anesthetic act directly on K^+-channels responsible for the maintenance of the resting potential in the myotube.

Thus, regardless of the mechanism by which tetracaine elicits Ca^{2+} release from intracellular stores,

it is safe to conclude that the initial phase of the $[Ca^{2+}]_i$ signal during the application of tetracaine, and possibly the $[Ca^{2+}]_i$ rise after the removal of the drug, are determined, at least in part, by the effects of the drug on V_m.

As to the effects of tetracaine on internal Ca^{2+} stores, the drug has been shown to interact with skinned muscle fibers [22], and SR vesicles in vitro releasing Ca^{2+} or blocking release depending on experimental conditions [23–25]. However, we found that in cultured rat myotubes the $[Ca^{2+}]_i$ transient elicited by tetracaine is not affected by the $[Ca^{2+}]_i$ level (60–330 nM) prior to the application of the drug. Furthermore, the drug increases the open-probability of the Ca^{2+} release channel reconstituted in planar lipid bilayers [26] and to interfere with ryanodine binding [27].

Procaine, known to inhibit the Ca^{2+}-induced release of Ca^{2+} mechanism (CIRC) in vertebrate muscle [13,14], was without noticeable effects on myotube resting $[Ca^{2+}]_i$ and inhibited the delayed component of the high $[K^+]_o$-induced $[Ca^{2+}]_i$ rise. We interpret this result to indicate that CIRC is not involved in the early phase of high $[K^+]_o$-induced $[Ca^{2+}]_i$ rise in cultured mature rat myotubes. Since the delayed component is substantially inhibited by procaine (2–5 mM), we tentatively propose that CIRC may be responsible for the second phase of the $[Ca^{2+}]_i$ rise elicited by high $[K^+]_o$ [13,14].

It should be mentioned here that in crustacean skeletal muscle tetracaine but not procaine blocks high $[K^+]_o$-induced contractures. Furthermore, under voltage clamp conditions, although inward Ca^{2+} currents were unaffected by these anesthetics, twitches were blocked [28]. Interestingly, inositol 1,4,5-trisphosphate ($InsP_3$) elicits contraction even in the presence of tetracaine in skinned barnacle muscle fibers [28]. This result demonstrates that the $InsP_3$ receptor on the SR membranes is not affected by tetracaine. Furthermore, tetracaine reversibly inhibited caffeine-induced contractures [29].

In conclusion, many of the features of E–C coupling which were established using micro-dissected skeletal muscle fibers are also present in mature myotubes. Thus, cultured rat myotubes constitute a useful model system in which to study the development of E–C coupling in mammalian skeletal muscle, particularly in lethal mutations, such as muscular dysgenesis, where the embryos survive just to birth [4].

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